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Acidic oligosaccharide sugar chain, a marine-derived oligosaccharide, activates human glial cell line-derived neurotrophic factor signaling☆

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Abstract

Gial derived neurotrophic factor (GDNF) modulates neuronal cell differentiation during development and protects against neurodegeneration by preventing apoptosis at maturity. GDNF's role in tissue maintenance has generated interest in the therapeutic potential of GDNF in treating neurological disorders such as Parkinson's disease. Heparan sulfate has been shown to be essential for GDNF signaling and altering the levels of heparan sulfate promotes or inhibits GDNF functional activity. To search for other oligosaccharides capable of modulating GDNF activity as potential therapeutic molecules, we investigated the effect of acidic oligosaccharide sugar chain (AOSC) and its sulfated derivative on GDNF induced neurotrophic events by using Western-blotting, immunofluorescence cell staining, and immunoprecipitation techniques in PC12 cells expressing the GDNF receptors GFRα1-Ret. AOSC significantly improved the neurite outgrowth and activated c-Ret phosphorylation in PC12-GFRα1-Ret cells, but its sulfated derivative inhibited GDNF activity. Studies to understand the opposing biological effects of AOSC and its sulfated derivative on GDNF activity demonstrated that reduced GDNF binding to PC12-GFRα1-Ret cell surface in the presence of the sulfated derivative likely suppressed GDNF activity as both AOSC and its sulfated derivatives had similar binding affinities to GDNF. This study illustrates the importance of oligosaccharide structure and charge on influencing GDNF activity and the potential use of oligosaccharides in modulating GDNF activity for therapeutic purposes.

Keywords: Glial cell line-derived neurotrophic factor (GDNF); Acidic oligosaccharide sugar chain (AOSC); Glycosaminoglycans (GAG); Heparin sulfate proteoglycan (HSPG); Neuroprotection

Glial cell line-derived neurotrophic factor (GDNF) is a distant member of the TGFβ-superfamily, which comprises an expanding list of multifunctional proteins serving as regulators of cell proliferation and differentiation. It is well established that GDNF exerts potent neuroprotective and regenerative activities against neurodegeneration. These neuroprotective features make the GDNF system a promising target for clinical manipulation [1,8]. Heparan sulfate (HS) is an acidic polysaccharide that is nearly ubiquitous on cell surface and in the extracellular matrix. It has been shown to bind to an increasing number of diverse cytokines [10]. Heparan sulfate proteoglycans (HSPGs) have been implicated in a wide variety of biological processes such as growth factor signaling, cell adhesion, wound healing, and tumor metastasis. Heparan sulfate glycosaminoglycans (GAGs) plays a direct role in GDNF signaling [2]. However, little is known about the modulation of exogenous GAGs mimetics in GDNF-associated functional events.

Acidic oligosaccharide sugar chain (AOSC), a marine-derived acidic oligosaccharide, was extracted from brown algae, Echlonia Kurome Okam, by depolymerization. It is rich in mannuronate blocks, with the average molecular weight of 1300 Da. Our previous studies have shown that AOSC attenuates neurotoxicity induced by both β-amyloid protein [7] and hydrogen superoxide (H_2O_2) in human neuroblastoma SHSY5Y cells in vitro [5]. Importantly, we further demonstrated that AOSC and its sulfate derivative can pass the blood–brain barrier via a GluT-1 transporter-involved mechanism [6]. Collectively, these findings hold promise for AOSC as a potential preventative or therapeutic neuroprotector against neurodegenerative disorders including Alzheimer's disease and Parkinson's disease.

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Therefore, the present study aimed to elucidate the possible involvement of AOSC in GDNF-mediated signaling events, and also to preliminarily identify the potential structural requirement of AOSC for its neuroprotective effect.

AOSC and its sulfate derivative were provided by Marine Drug and Food Institute at Ocean University of China. The CM5 biosensor chip was purchased from BIAcore (Uppsala, Sweden). Bacterial expression plasmid pET28a(+)-rhGDNF and PC12-GFRα1-Ret stable transfected cell line were generous gifts from the Department of Neurobiology, Second Military Medical University. Anti-phosphotyrosine monoclonal antibody pY99, anti-Ret goat polyclonal C-20 antibody, anti-GDNF mouse monoclonal antibody B8, FITC-conjugated IgG and rhGDNF were from Santa Cruz Biotechnology. All other chemicals were purchased from BBI.

The cells were cultured in DMEM containing 10% FBS and differentiation assays were performed as previously described [14]. PC12-GFRα1-Ret cells were incubated with or without GDNF in combination with either AOSC or its sulfated derivative. GDNF isolation and functional assays were carried out as previously described [14,13]. Differentiation was defined as cells developing neurite processes with length more than twice the diameter of the cell body. Cells meeting this criteria were counted as positive. A minimum of a hundred cells was examined in four fields for each data point and expressed as the mean ± S.E. (Table 1).

The biotinylated AOSC or its sulfate derivative was immobilized onto streptavidin-CM5 sensor chip surface. The immobilization procedures were carried out at 25 °C with a constant flow rate of 5 μl /min HBS-EP buffer (0.01 M HEPES, 0.15 M NaCl, pH 7.4). After stabilization, rhGDNF were injected over the AOSC-immobilized or derivative-immobilized CM5 chips, respectively. The sensor chips were then regenerated by washing with 2 M NaCl. To correct for non-specific binding and bulk refractive index change, blank channel without AOSC or derivative were used and run simultaneously. Sensorgrams for all binding interactions were recorded in real time and analyzed after subtracting that from the blank channel. Changes in mass due to the binding response were recorded as resonance units (Ru).

PC12-GFRα1-Ret cells were seeded at 2×10^5 cells per well using 6-well plates and 24 h later incubated with or without 200 ng/ml GDNF and in combination with either 10 μg/ml SPMG or 10 μg/ml AOSC. After 48 h, 10^6 PC12-GFRα1-Ret cells were collected and washed with PBS. Cells were then incubated with anti-GDNF antibody for 3 h at room temperature followed by FITC-conjugated IgG. Antibody labeled cells were washed three times with PBS prior to flow cytometry analysis. Data were acquired with Cellquest II software by using fluorescence triggering in the FL1 channel to gate on PC12-GFRα1-Ret cell populations based on the FSC/SSC light scattering in a mode of 10000 gated events. The mean fluorescence intensity reflects GDNF binding to GFRα1 and Ret receptor anchor on the cell surface.

Table 1
The kinetic parameters of the interactions between AOSC, derivative and GDNF

Ligand–analyte	k_a (1/M s)	k_d (1/s)	K_A (1/M)	K_D (M)
AOSC–GDNF	26.6	4.24e-3	6.28e3	1.59e-4
Derivative–GDNF	2.01	6.76e-5	2.98e4	3.36e-5

The experiment was carried out at 25 °C with a constant flow rate of 5 μl/min HBS–EP buffer.

Confluent PC12-GFRα1-Ret cells were serum-starved for 2 h before ligand treatment. Cells were then stimulated with GDNF, AOSC, and AOSC derivative alone or GDNF in combination with either AOSC or AOSC derivative for 15 min at 37 °C. Afterwards cells were washed with ice-cold PBS and lysed in lysis buffer [12]. Lysate protein concentrations were quantitated using the Bio-Rad protein assay using BSA as the standard. Aliquots were taken from each lysate for protein phosphorylation assay as described previously [12]. Briefly, polyclonal goat anti-Ret antibodies were covalently crosslinked to protein G-agarose and used for immunoprecipitation. Monoclonal mouse anti-phosphotyrosine and polyclonal goat anti-Ret antibodies were used for immunoblotting to detect Ret activation.

In order to investigate the effects of AOSC and its derivative on PC12-GFRα1-Ret differentiation induced by GDNF, neurite outgrowth was quantitated by counting the number of cells that developed neurite processes of length more than twice the diameter of the cell body. GDNF significantly promoted neurite outgrowth of PC12-GFRα1-Ret but not in PC12 cells lacking the GDNF receptors ($p < 0.01$, Fig. 1B and G). Interestingly, AOSC further promoted the neurite outgrowth of PC12-GFRα1-Ret cells induced by GDNF as compared with the GDNF-treated alone group ($p < 0.01$, Fig. 1D and G). However, the sulfate derivative of AOSC inhibited GDNF-associated PC12-GFRα1-Ret neurite outgrowth as compared with the GDNF alone group ($p < 0.01$, Fig. 1C and G). Together the results suggest that AOSC potentiates GDNF induced neural cell differentiation whereas the sulfated derivative appeared to attenuate differentiation and that GDNF requires its receptors, GFRα1 and Ret, to induce PC12 neurite outgrowth. We also compared the quality of our rhGDNF isolated from *E. Coli* to the rhGDNF purchased from Santa Cruz Inc. The in lab generated and commercial rhGDNF exhibited similar promoting function on PC12-GFRα1-Ret neurite outgrowth (data not shown), justifying and validating the use of our rhGNDF instead of the more expensive commercial product.

Recent studies have demonstrated that heparan sulfate GAGs can promote the binding of GDNF to its receptor via binding to GDNF, and thus play a direct role in GDNF-triggered signaling [2]. In fact, AOSC and its sulfate derivative are both GAGs mimetics, so we want to address whether the influence of AOSC and its sulfate derivative on GDNF-induced neurite outgrowth of PC12-GFRα1-Ret cells and subsequent signaling might be due to its direct association with GDNF.SPR assay is increasingly recognized to be an accurate method for identifying the interaction between sugar and protein [12,3]. Therefore, we used SPR to assess the 'real-time' recognition of biomolecular interactions. The results showed that both AOSC and its sulfate derivative bind to GDNF in a low affinity manner, with K_D of 1.93e−4 and 4.64e−5, respectively (Table 1, Fig. 2B), and the binding

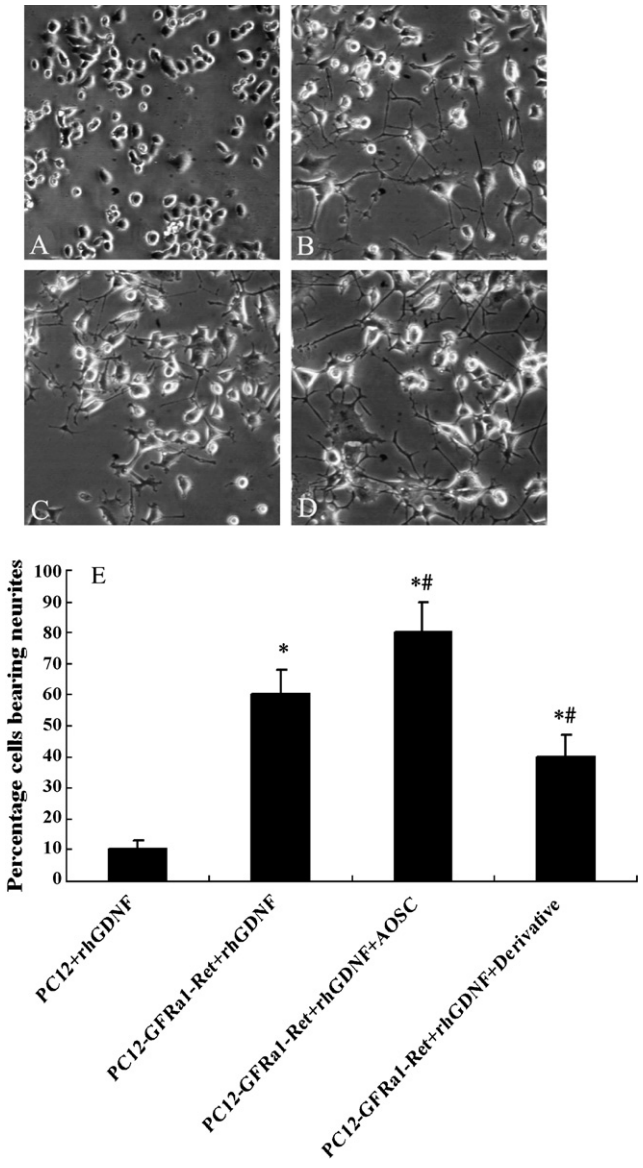

Fig. 1. AOSC promotes, but its sulfated derivative hinders the neurite outgrowth induced by GDNF in PC12-GFRα1-Ret cells. (A) Wild type PC12 cells stimulated with 200 ng/ml rhGDNF. (B) PC12-GFRα1-Ret cells stimulated with 200 ng/ml rhGDNF. (C) PC12-GFRα1-Ret cells stimulated with 200 ng/ml rhGDNF and 10 μg/ml derivative. (D) PC12-GFRα1-Ret cells stimulated with 200 ng/ml rhGDNF and 10 μg/ml AOSC. (G) The statistics of effects of AOSC and its sulfated derivative on PC12-GFRα1-Ret cells neurite outgrowth induced by GDNF. Each value represents the mean ± S.D. from three independent experiments. (*) $p < 0.01$ compared with wild type PC12 cells. (#) $p < 0.01$ compared with GFRα1-Ret-PC12 cells stimulated with rhGDNF.

profile of GDNF with AOSC and its sulfate derivative are nearly the same (Fig. 2A).

Although both AOSC and its sulfated derivative exhibited low binding affinity for GDNF, they displayed contrasting biological effects on neurite outgrowth induced by GDNF. Thus, we examined whether the diametrically opposed biological effects reflected differences in influencing GDNF binding to its cell surface receptor. Similar levels of GDNF bound to PC12-GFRα1-Ret cell in the presence or absence of AOSC (Fig. 3, compare d and c) but lower levels of GDNF bound in the presence of its derivative (Fig. 3 compare b and d), indicating AOSC did not affect GDNF binding to the PC12-GFRα1-Ret cell surface whereas its derivative reduced but did not completely inhibit GDNF binding. Further studies are required to determine whether the association of the sulfated derivative with GDNF hinders GDNF binding to its receptors, GFRα1 or Ret, or whether the interaction of the sulfated derivative with the receptors, GFRα1 or Ret, retards GDNF-receptor binding.

Given the opposing influences of AOSC and its derivative on GDNF binding to cell surface receptors, we investigated if GDNF cell surface binding directly correlated with GDNF receptor cell signaling. Another measure of GDNF and its receptor interaction is to monitor GDNF cell signaling through the status of Ret phosphorylation. As shown in Fig. 4A, Western-blots with anti-phosphotyrosine antibody illustrate that AOSC promoted GDNF-induced Ret phosphorylation in PC12-GFRα1-Ret cells, concordant with GDNF cell surface binding. Likewise, its sulfate derivative reduced Ret phosphorylation as compared with PC12-GFRα1-Ret cells stimulated with GDNF. AOSC and its sulfated derivative alone had no influence on Ret phosphorylation in PC12-GFRα1-Ret cells (Fig. 4B). Thus, AOSC appears to facilitate neurite outgrowth by potentiating GDNF initiated signaling through Ret receptor phosphorylation and its sulfated derivative inhibits this cell signaling process.

GDNF plays a vital role in the development and maintenance of the nervous system and in the morphogenesis of the kidney and testes. Understanding the details of how GDNF interacts with its receptors is therefore an interest in the study of embryogenesis, organogenesis, development and neurobiology. GDNF signaling requires the GFRα1 and Ret receptor complex. Additionally, studies have demonstrated oligosaccharide chains such as heparin sulfate and GAGs also have a role in modulating GDNF biological activity [3,11]. Other growth factors known to depend on GAGs for signaling include FGF and HGF [15]. GDNF signalling requires heparan but not dermatan or chondroitin sulfates [9]. One function of GDNF binding to heparan sulfate with low affinity might simply be to concentrate the growth factor in the vicinity of its high affinity receptors. By contrast, in cells that express endogenous HSPGs, some exogenously administrated GAGs may inhibit rather than stimulate GDNF signaling, presumably by perturbing the interactions of cellular HSPGs with GDNF/ GDNFR. In the present studies, we found that AOSC significantly promoted the neurite outgrowth and subsequently potentiated GDNF activated Ret phosphorylation in PC12-GFRα1-Ret cells. However, the sulfate derivative of AOSC inhibited GDNF-induced neurite outgrowth and signaling cascades in PC12-GFRα1-Ret cells. The discrepancy of AOSC and its sulfated derivative on the biological activity of GDNF likely reflects the inhibition of GDNF binding to the cell surface in the presence of the sulfated derivative but not with AOSC. At present it is unclear whether the interaction of the sulfated derivate with GDNF or GDNF receptors, GFRα1 and Ret, hinders GDNF binding. Given that AOSC and sulfated derivative alone did not change the Ret phosphorylation in PC12-GFRα1-Ret cells, these oligosaccharides modulate neurite growth by

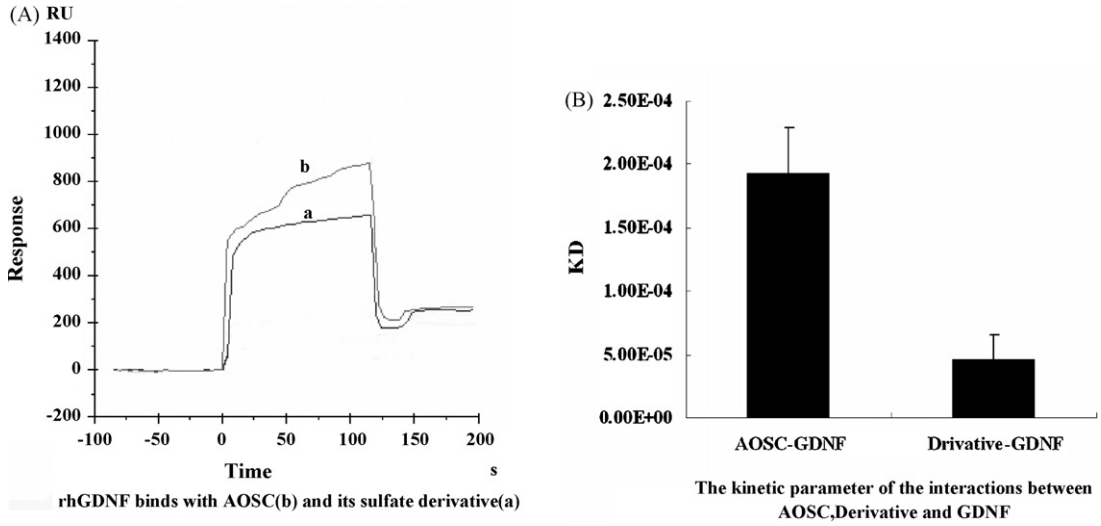

Fig. 2. Both AOSC and its sulfated derivative interact with GDNF by SPR assay. (A) Binding of rhGDNF to AOSC (b) or its derivative (a) was assayed using the sensor chip surface technology. The *Y*-axis represents the binding resonance unit.

influencing GDNF signaling in PC12-GFRα1-Ret cells through Ret phosphorylation.

Sugar–protein interactions are specified by charge, size, structure and composition of the oligosaccharide. Sulfation is a common modification that can affect the physical properties of oligosaccharides and thus the extent and distribution of sulfation on the sugar backbone can have adverse or beneficial affects on sugar-protein interactions [16]. Molecular details of the interactions whereby PGs interfere with or promote neurite growth are still largely unknown. GDNF only displays high specificity for particular sugar sequences of some GAGs. Yet little is known about the binding specificity of GDNF for different types of heparan sulfate, except for the fact that some sulfated residues must be involved. Studies have shown that 2-O-sulfation is much more important to heparin's ability to interact with GDNF signaling than are 6-*O*- or *N*-sulfations [2,4]. In the present studies, we found that both AOSC and its sulfate derivative, though displaying a similar binding profile with GDNF, have opposite biological effects on generating de novo neural processes. Structurally, AOSC is comprised of a mannuronate backbone with hydroxyl groups while its sulfated derivative is modified at the C-2 and partially C-3 with a sulfate moiety. Given AOSC and its sulfated derivative differ only in the extent of their sulfate content, it is likely that the sulfate group accounts for the discrepancy in their corresponding neurological functions. It is commonly accepted that the periodic deposition of electron-dense sulfate along the sugar chain is critical for competition with endogenous heparan sulfate to bind to GDNF and thus favoring for the arrest of the subsequent biological cascades. This notion can greatly account for the antagonizing potency of the sulfate derivative on GDNF-associated neurite outgrowth and Ret phosphorylation, with C2/C3 sulfate moiety being of critical importance. However, the facilitative action of AOSC on GDNF-induced neurotrophic events is likely attributed to the carboxyl groups along the AOSC, allowing low but detectable binding affinity for GDNF which might subsequently promote the oligomerization and presentation of GDNF to its receptor.

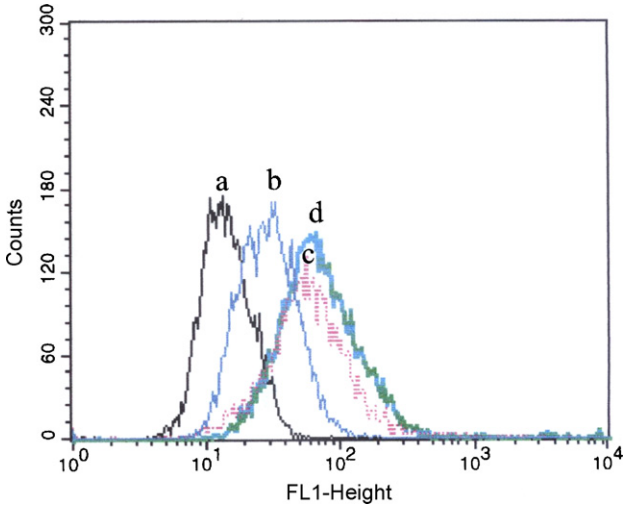

Fig. 3. AOSC does not influence, but its sulfated derivative reduces GDNF binding to the PC12-GFRα1-Ret cell surface. (a) Wild type PC12 stimulated with 200 ng/ml rhGDNF (control); (b) PC12-GFRα1-Ret cells stimulated with 200 ng/ml rhGDNF and 10 μg/ml derivative; (c) PC12-GFRα1-Ret cells stimulated with 200 ng/ml rhGDNF and 10 μg/ml AOSC; (d) PC12-GFRα1-Ret cells stimulated with 200 ng/ml rhGDNF. This experiment was repeated three times.

In conclusion, AOSC can increase GDNF-associated signaling events, suggesting that AOSC can be clinically beneficial by enhancing the neuroprotective function of GDNF. A better understanding of how AOSC increases, but its sulfated derivative inhibits, GDNF-associated axon extension and subsequent signaling cascades will be crucial for the elucidation of the sugar-based molecular mechanisms underlying cell growth, differentiation and survival of neuronal cells, and thus provide the basis to develop therapeutic strategies for human disease after a lesion in the central nervous system.

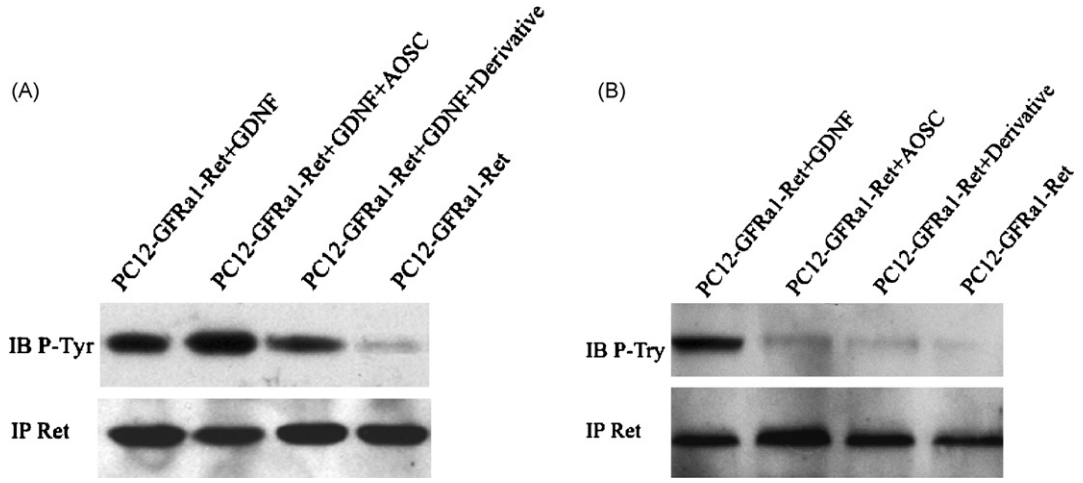

Fig. 4. AOSC activates, but its sulfated derivative inactivates Ret phosphorylation induced by GDNF. PC12-GFRα1-Ret cells were treated with AOSC or the sulfated derivative in the presence (A) or absence of GDNF (B). Cell lysates were immunoprecipitated with anti Ret antibody and then immunoblotted for phosphorylated Ret with anti phosphotyrosine antibody. The lower panels show aliquots of cell lysates immunoprecipitated by anti-Ret antibodies.

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